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Effect of Arachidonic Acid on Twitch Tension of the Rat Phrenic Nerve-Diaphragm[1]

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ABSTRACT

Recent studies have demonstrated that unsaturated fatty acids are involved in the regulation of neuroeffector function. I have extended these studies by examining the effect of arachidonic acid on neuromuscular function *in vitro* using the rat phrenic nerve-diaphragm preparation. Arachidonic acid caused a time- and dose-dependent reduction in indirectly stimulated twitch tension, but had no effect on directly stimulated twitch tension. Linoleic acid and linolenic acid also reduced indirectly stimulated twitch tension, whereas stearic acid, oleic acid and arachidic acid had no effect. None of three blockers of arachidonic acid metabolism, the cyclooxygenase inhibitor indomethacin, the lipoxygenase inhibitor nordihydroguaiaretic acid or the cytochrome P-450 inhibitor ketoconazole, altered the effect of arachidonic acid on twitch tension. The free radical scavenger superoxide dismutase eliminated the inhibitory effect of arachidonic acid on twitch tension, suggesting that superoxide anion played a role in arachidonic acid's action.

The role of unsaturated fatty acids, particularly arachidonic acid and its metabolites, in the regulation of neuroeffector function is becoming increasingly apparent (Gustafsson, 1989; Ordway *et al.*, 1991; Piomelli and Greengard, 1990; Shimizu and Wolfe, 1990). In mammalian systems, arachidonic acid itself has been implicated in the modulation of synaptosomal amino acid flux (Chan *et al.*, 1983; Freeman *et al.*, 1990; Lynch and Voss, 1990; Rhoads *et al.*, 1983a,b), choline uptake (Boksa *et al.*, 1988; Saltarelli *et al.*, 1990) and calcium uptake (Kandasamy and Hunt, 1990). It has also been reported to modulate calcium current (Keyser and Alger, 1990) and synaptic transmission (Carlen *et al.*, 1989; Williams *et al.*, 1989) in hippocampal neurons. This article extends these studies by examining the effect of arachidonic acid on neuromuscular function in a mammalian neuroskeletomuscular preparation, that of the phrenic nerve-diaphragm of the rat.

Methods

Materials. All fatty acids, indomethacin and nordihydroguaiaretic acid were purchased from Sigma Chemical Co. (St. Louis, MO). (+)-Tubocurarine chloride was purchased from QUAD Pharmaceuticals Inc. (Indianapolis, IN) and SKF-525A hydrochloride from Research Biochemicals Inc. (Natick, MA)

Twitch tension measurements. Male Sprague-Dawley rats (80–120 g; Harlan Sprague-Dawley, Inc., Frederick, MD) were housed four per cage, maintained on a 12-h light/dark (6:00 P.M. to 6:00 A.M.) cycle and allowed free access to food and water. Their care and use were in compliance with the Animal Welfare Act (U.S.) and the National Institutes of Health's Guide for the Care and Use of Laboratory Animals. Rats were anesthesized with gaseous carbon dioxide and decapitated. Dissection of phrenic nerve-hemidiaphragms and measurement of twitch tension were performed according to Kitchen (1984) after Bulbring (1946), with phrenic nerve electrodes, transducers and paper chart recorder from Harvard Apparatus (South Natick, MA) and a four-channel stimulator from Grass Instruments (Quincy, MA). Experiments were performed in both 100-ml glass organ baths (Harvard Apparatus) and 100-ml Teflon beakers (Scientific Products, McGaw Park, IL). Results were comparable between the two containers, but the Teflon beakers had less build up of fatty material and were easier to clean. The organ bath solution (60 ml) was 1.8 mM $CaCl_2$, 11 mM glucose, 5.0 mM KCl, 0.50 mM $MgSO_4$, 24 mM $NaHCO_3$, 137 mM NaCl and 1.0 mM NaH_2PO_4 (pH 7.3). The solution was oxygenated with 95% O_2/5% CO_2. All water-insoluble compounds were dissolved in dimethyl sulfoxide [no more than 0.12% (v/v)]. When compared to organ bath solution, dimethyl sulfoxide had no effect on twitch tension over the time periods studied (data not shown). Unless specifically noted, all stimulation was indirect using the following parameters: 7.6 V amplitude, 250 ohm input impedance, 0.25 msec pulse duration and 1 Hz. When used, direct stimulation was performed in the presence of 5 μM (+)tubocurarine chloride under the same conditions used for indirect stimulation except that the stimulation parameters were 10 V amplitude, 25 ohm input impedance, 2.5 msec pulse duration and 1 Hz. All stimulation was maximal and continuous during the experiments. The height of pen travel was used as the measure of twitch tension.

Statistical analyses. One-way analysis of variance was performed to evaluate overall effects. Fisher's least significance difference test was used for *post hoc* group comparisons when there was a significant overall effect. $P < .05$ (two-tailed) was required for significance. N was 4 to 11 diaphragms for each group with the goal to have the S.E.M. < 10% of the corresponding mean.

Results

Dose effect of arachidonic acid on twitch tension. I added various doses of arachidonic acid to phrenic nerve-

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[1] A preliminary account of this research was presented in November 1991 at the 10th World Congress on Animal, Plant, and Microbial Toxins in Singapore (Crosland, 1992).

diaphragm preparations and observed the resultant changes in twitch tension (fig. 1). All doses produced an immediate reduction in twitch tension in the first 7.5 min. Thereafter, the lower doses (3.2 and 10 μM) induced a smaller rate of decline in twitch tension for about 1 h, followed by a further decrease in the rate of decline. After 7.5 min, two intermediate doses (32 and 180 μM) produced a smaller rate of decline in twitch tension which remained more or less constant for the remainder of the experiment. One hundred μM arachidonic acid produced a rather rapid reduction in twitch tension for up to 15 min, followed by a smaller rate of decline for 1 h, which was followed by an almost leveling off in twitch tension for the remaining 45 min. Three hundred twenty μM arachidonic acid followed the pattern of the intermediate doses for 30 min, and then induced a rapid, constant rate of decline in twitch tension for the remainder of the experiment.

Although there were significant reductions in twitch tension after only 7.5 min of arachidonic acid treatment (P = .0044), it appeared that 2 h were required to allow the effects of the various doses of arachidonic acid to be fully expressed, and twitch tension at this time point was used to measure effects in subsequent experiments. The dose effect of arachidonic acid on twitch tension after 2 h is depicted in the inset of figure 1. The dose effect exhibited an ED_{50} of 130 μM with a Hill coefficient of 1.

In order to determine the reversibility of the arachidonic acid-induced reduction in twitch tension, I replaced the organ bath solution containing arachidonic acid with solution lacking arachidonic acid after various periods of incubation (fig. 2). Reduction in twitch tension measured at 2 h depended on the length of time of incubation with arachidonic acid and was partially reversible after up to 60 min of incubation.

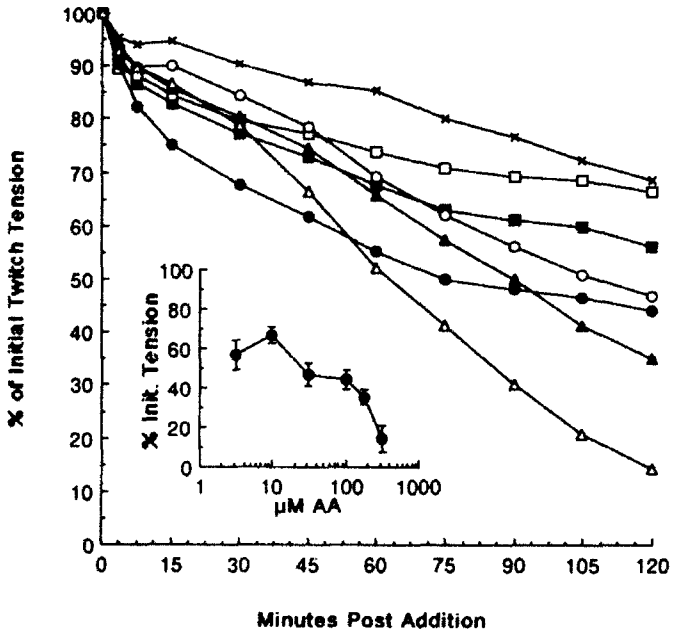

Fig. 1. Effect of various doses of arachidonic acid on twitch tension. Phrenic nerve-diaphragms were stimulated in the presence of various concentrations of arachidonic acid. Dimethyl sulfoxide only (×), 3.2 μM (■), 10 μM (□), 32 μM (○), 100 μM (●), 180 μM (▲), 320 μM (△). Values are means, S.E.M. omitted for clarity. Statistics at 2 h: P (over all doses) = .0001. Significant *post hoc* comparisons *vs.* dimethyl sulfoxide: 32 μM, 100 μM, 180 μM, 320 μM. Inset: Dose effect of arachidonic acid on twitch tension after 2 h of incubation. Values are mean ± S.E.M.

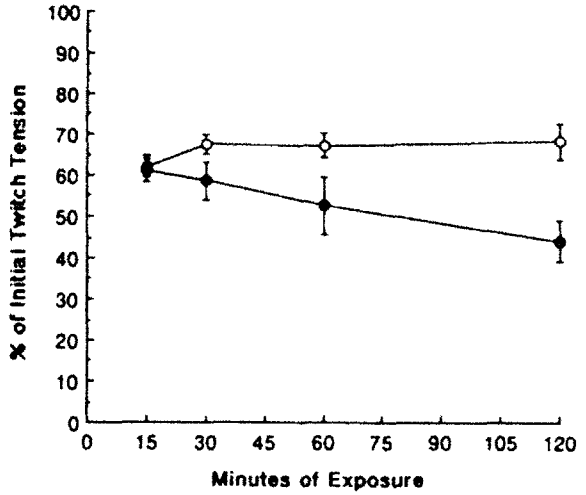

Fig. 2. Reversibility of arachidonic acid-induced reduction in twitch tension. Diaphragms were stimulated in the presence of dimethyl sulfoxide or 100 μM arachidonic acid. At the times indicated, the organ bath solution was removed and replaced with organ bath solution containing neither dimethyl sulfoxide nor arachidonic acid, and stimulation continued for the remainder of the 2-h incubation. Dimethyl sulfoxide (○), arachidonic acid (●). The data depicted are twitch tension after 2 h expressed as a percentage of the twitch tension at 0 h. Values are mean ± S.E.M. P (arachidonic acid *vs.* dimethyl sulfoxide): 15 min, .82; 30 min, .13; 60 min, .077; 120 min, .002.

Does arachidonic acid affect direct stimulation of muscle? Figure 3 depicts the effect of arachidonic acid on direct stimulation of the diaphragm. There was no difference in the effect of arachidonic acid when compared to either organ bath solution or dimethyl sulfoxide.

At the end of 2 h of incubation, neostigmine (3.3 μM final concentration) was added to the dimethyl sulfoxide and arachidonic acid only-treated diaphragms of the ketoconazole experiment of table 1. After approximately 7.5 min, twitch tension increased in both groups and then declined. The initial rate of increase in twitch tension (expressed as percent of initial twitch tension/sec) was 60% less in diaphragms treated with arachidonic acid than in control diaphragms (mean ± S.E.M.: 0.20 ± 0.04 *vs.* 0.50 ± 0.07, P = .0015).

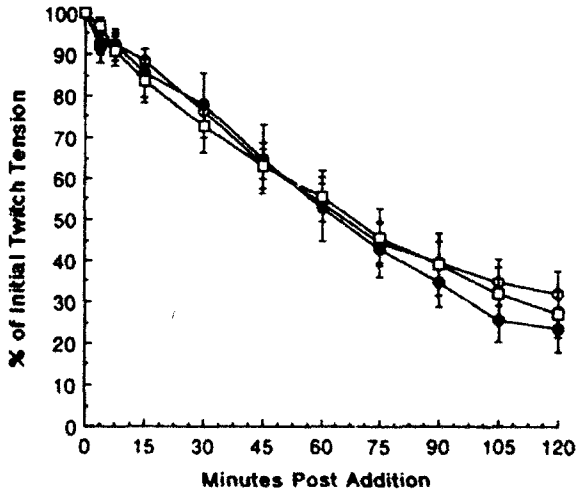

Fig. 3. Effect of arachidonic acid on direct stimulation of rat diaphragm. Diaphragms were stimulated directly for 2 h in the presence of organ bath solution (□), dimethyl sulfoxide (○) or 100 μM arachidonic acid (●). Values are mean ± S.E.M. Statistics at 2 h: P (over all groups) = .55.

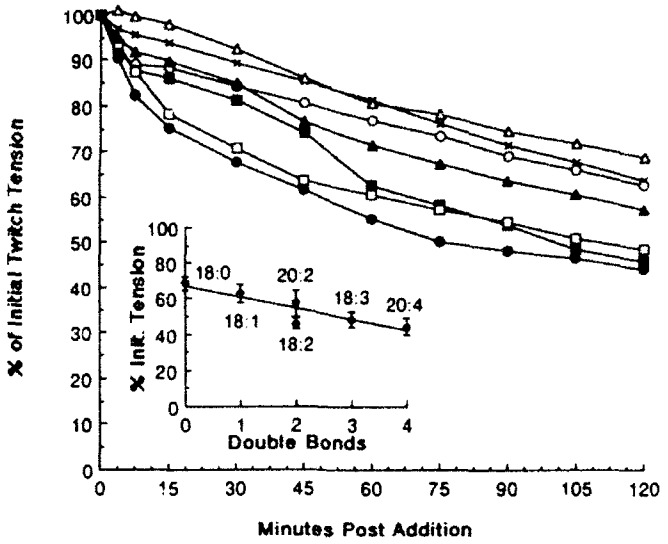

Fig. 4. Effect of various fatty acids on twitch tension. Various fatty acids (100 μM final concentration) were incubated separately for 2 h with stimulated diaphragms. Dimethyl sulfoxide (×), stearic acid (18:0) (△), oleic acid (18:1) (○), linoleic acid (18:2) (■), linolenic acid (18:3) (□), arachidic acid (20:2) (▲), arachidonic acid (20:4) (●). The arachidonic acid data are the same as those shown in figure 1. Values are means S.E.M. omitted for clarity. Statistics at 2 h: P (over all fatty acids) = .0041. Significant *post hoc* comparisons *vs.* dimethyl sulfoxide: linoleic acid, linolenic acid, arachidonic acid. Inset: Correlation of twitch tension with degree of unsaturation. The means ± S.E.M. of the twitch tension data of the 2-h time points were plotted against the number of double bonds in the corresponding fatty acids. The rank order correlation was −0.90 (P = .044).

Effect of various fatty acids on twitch tension. I tested various fatty acids (100 μM) for their effects on twitch tension (fig. 4). While stearic acid slightly increased twitch tension compared to dimethyl sulfoxide alone, some fatty acids caused a significant reduction in twitch tension in as little as 3.75 min (P = .0003). Oleic acid and arachidonic acid caused fairly rapid rates of decline in twitch tension for the first 7.5 min, followed by less rapid, constant rates of decline for the remainder of the experiment. Linoleic acid caused an initial, rapid rate of decline in twitch tension for 7.5 min, followed by a rate of decline similar to that of oleic acid and arachidonic acid for approximately 30 min, followed by another fairly rapid rate of decline for 15 min, and finally by a slower rate of decline in twitch tension for the remaining 60 min. Linolenic acid produced a temporal pattern of twitch tension similar to that of arachidonic acid.

Linoleic acid, linolenic acid and arachidonic acid caused significant reductions in twitch tension after 2 h. The decline in twitch tension after 2 h caused by the various fatty acids correlated well with the number of double bonds in the fatty acids (fig. 4 inset). The more unsaturated a fatty acid was, the more of a decline in twitch tension it caused.

Effect of inhibitors of arachidonic acid metabolism on arachidonic acid's effect on twitch tension. Inhibitors of arachidonic acid metabolism could help to determine whether or not the effect of arachidonic acid on twitch tension was mediated by a metabolite of arachidonic acid. Three metabolic enzymes which act on arachidonic acid are cyclooxygenase, lipoxygenase and cytochrome P-450 (Needleman *et al.*, 1986). Indomethacin (an inhibitor of cyclooxygenase) had no effect on twitch tension by itself and had no effect on arachidonic acid's reduction of twitch tension (table 1). The lipoxygenase inhibitor nordihydroguaiaretic acid was also ineffective, although it tended to reduce twitch tension by itself and also tended to alleviate the reduction in twitch tension caused by arachidonic acid (table 1). The cytochrome P-450 inhibitor ketoconazole had no effect itself on twitch tension (table 1). It also did not alleviate arachidonic acid's reduction of twitch tension, although it tended in that direction. A second inhibitor of cytochrome P-450 (SKF-525A, 50 μM) by itself completely eliminated twitch tension within 15 min (data not shown).

TABLE 1

Effect of various agents on arachidonic acid-induced reduction in twitch tension

For indomethacin, nordihydroguaiaretic acid and ketoconazole, diaphragms were preincubated for 15 min with agent (dissolved in no more than 12 μl of dimethyl sulfoxide) or dimethyl sulfoxide. For superoxide dismutase, diaphragms were preincubated with agent (in 60 μl of organ bath solution) or organ bath solution. All preincubations were followed by incubation for 2 h with 100 μM arachidonic acid (dissolved in 60 μl of dimethyl sulfoxide) or dimethyl sulfoxide. Values are mean ± S.E.M. of twitch tension after 2 h of treatment expressed as percent of twitch tension at 0 h. Statistical comparisons were made horizontally: P (overall for each agent): indomethacin, .0003; nordihydroguaiaretic acid, .029; ketoconazole, .0023; superoxide dismutase, .0011. The pairwise comparisons tested were dimethyl sulfoxide *vs.* each group and arachidonic acid alone *vs.* agent + arachidonic acid.

Agent	Dimethyl Sulfoxide	Agent Alone	Arachidonic Acid Alone	Agent + Arachidonic Acid
30 μM indomethacin	64 ± 4	62 ± 4	43 ± 3[a]	45 ± 4[a]
30 μM nordihydroguaiaretic acid	63 ± 3	55 ± 6	44 ± 4[a]	51 ± 4
10 μM ketoconazole	66 ± 3	66 ± 2	46 ± 4[a]	55 ± 5
90 U/ml superoxide dismutase	67 ± 3	63 ± 1	46 ± 4[a]	59 ± 4[b]

[a] Significantly different from dimethyl sulfoxide
[b] Significantly different from arachidonic acid alone

Effect of superoxide dismutase on arachidonic acid's effect on twitch tension. The free radical scavenger superoxide dismutase reduces levels of superoxide anion by converting it to oxygen and hydrogen peroxide. Superoxide dismutase eliminated arachidonic acid's effect on twitch tension, but had no effect of its own (table 1). Superoxide dismutase (90 U/ml) incubated for 10 min in a boiling water bath did not alter arachidonic acid's effect on twitch tension (data not shown).

Discussion

Arachidonic acid caused a time- and dose-dependent reduction in twitch tension of the phrenic nerve-diaphragm of the rat (fig. 1). The effective doses of arachidonic acid and its time course of action were comparable to those which modulate other physiological processes (Boksa *et al.*, 1988; Carlen *et al.*, 1989; Chan *et al.*, 1988; Keyser and Alger, 1990; Maruyama, 1990). There were two indications that the effect of arachidonic acid on twitch tension was relatively specific and nondisruptive. First, the effect was partially reversible after up to 60 min of incubation (fig. 2). Second, several other fatty acids had no effect on twitch tension (fig. 4), simultaneously demonstrating the specificity of arachidonic acid's action and lessening its possibility of having had a disruptive (*e.g.*, detergent) effect. Twitch tension elicited by direct stimulation of the muscle was not affected by arachidonic acid (fig. 3), demonstrating that arachidonic acid did not affect excitation-contraction coupling or contraction itself. Buttressing this conclusion was the effect of neostigmine. Neostigmine inhibits acetylcholinesterase, resulting in a buildup of acetylcholine in solution, particularly in the region of the endplate. In arachidonic acid-treated dia-

phragms, neostigmine caused an initial increase in twitch tension which was 60% less than that caused by neostigmine in dimethyl sulfoxide-treated diaphragms. Because acetylcholine affects muscle contraction only through interaction with postsynaptic acetylcholine receptors, arachidonic acid must affect neuromuscular function at a step(s) preceeding excitation-contraction coupling and contraction.

I endeavored to determine whether metabolites of arachidonic acid could have mediated its effect. Arachidonic acid is metabolized along three pathways which start with three separate enzymes: cyclooxygenase, lipoxygenase and cytochrome P-450. There exist inhibitors for each enzyme which can be used to block its respective pathway, thereby allowing the determination of whether or not metabolites of that pathway mediate the effect of arachidonic acid. The cyclooxygenase inhibitor indomethacin had no effect on arachidonic acid-induced decrease in twitch tension (table 1), absolving cyclooxygenase pathway metabolites of responsibility for arachidonic acid's effect. Although the lipoxygenase inhibitor nordihydroguaiaretic acid and the cytochrome P-450 inhibitor ketoconazole had no significant effect on arachidonic acid-induced reduction of twitch tension, both tended to attenuate the reduction (table 1). Based on the effect of these inhibitors in other systems, the data suggest that metabolites of arachidonic acid played little or no role in the effect of arachidonic acid on twitch tension.

Superoxide anion can be produced concurrently with the metabolism of arachidonic acid, and recent evidence implicates the superoxide anion as a mediator of some of the effects of arachidonic acid on physiological processes (Keyser and Alger, 1990; Puig-Parellada *et al.*, 1991). In the present study, the free radical scavenger superoxide dismutase eliminated the effect of arachidonic acid on twitch tension (table 1), suggesting that superoxide anion was necessary for arachidonic acid's effect. Three likely roles for superoxide anion are that it reduced twitch tension itself, that it acted through an intermediate such as hydroxyl radical and/or that it reacted with another compound such as arachidonic acid, with the resulting product causing the reduction in twitch tension. In the first two cases, arachidonic acid would appear to have been required to produce the superoxide anion. In the latter case, arachidonic acid could have reacted with endogenous superoxide anion.

The present research implicating superoxide anion in arachidonic acid's effect on twitch tension is reminiscent of research by Chan *et al.* (1988) on rat cerebral cortical astrocytes. These investigators showed that arachidonic acid and other polyunsaturated fatty acids could generate superoxide anion. For the fatty acids they tested which I also tested, they observed the same rank order of effectiveness in producing superoxide anion as I observed in reducing twitch tension (arachidonic > linolenic > linoleic > oleic = 0) raising the possibility that in my study the effect of the fatty acids other than arachidonic acid could also have involved superoxide anion.

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